Example 8. Demonstration of Hu-Asp2 β- Secretase Activity in Cultured Cells

Several mutations in APP associated with early onset Alzheimer's disease have been shown to alter Aβ peptide processing. These flank the N- and C-terminal cleavage sites that release A□ from APP. These cleavage sites are referred to as the β-secretase and γ-secretase cleavage sites, respectively. Cleavage of APP at the β-secretase site creates a C-terminal fragment of APP containing 99 amino acids of 11,145 daltons molecular weight. The Swedish KM→NL mutation immediately upstream of the β-secretase cleavage site causes a general increase in production of both the 1-40 and 1-42 amino acid forms of A□ peptide The London VF mutation (V717→F in the APP770 isoform) has little effect on total A□ peptide production, but appears to preferentially increase the percentage of the longer 1-42 amino acid form of A□ peptide by affecting the choice of γ-secretase cleavage site used during APP processing. Thus, we sought to determine if these mutations altered the amount and type of A□ peptide produced by cultured cells cotransfected with a construct directing expression of Hu-Asp2.

Two experiments were performed which demonstrate Hu-Asp2 β-secretase activity in cultured cells In the first experiment, treatment of HEK125.3 cells with antisense oligomers directed against Hu-Asp2 transcripts as described in Example 7 was found to decrease the amount of the C-terminal fragment of APP created by β-secretase cleavage (CTF99) (Figure 9). Thus shows that Hu-Asp2 acts directly or indirectly to facilitate β-secretase cleavage. In the second experiment, increased expression of Hu-Asp2 in transfected mouse Neuro2A cells is shown to increase accumulation of the CTF99 β-secretase cleavage fragment (Figure 10). This increase is seen most easily when a mutant APP-KK clone containing a C-terminal di-lysine motif is used for transfection. A further increase is seen when Hu-Asp2 is cotransfected with APP-Sw-KK containing the Swedish mutation KM →NL. The Swedish mutation is known to increase cleavage of APP by the β-secretase.

A second set of experiments demonstrate Hu-Asp2 facilitates γ-secretase activity in cotransfection experiments with human embryonic kidney HEK293 cells. Cotransfection of Hu-Asp2 with an APP-KK clone greatly increases production and release of soluble Aβ1-40 and Aβ1-42 peptides from HEK293 cells. There is a proportionately greater increase in the release of Aβ1-42. A further increase in production of Aβ1-42 is seen when Hu-Asp2 is cotransfected with APP-VF (SEQ ID No. 13 [nucleotide] and SEQ ID No. 14 [amino acid]) or APP-VF-KK SEQ ID No. 19 [nucleotide] and SEQ ID No. 20 [amino acid]) clones containing the London mutation V717→F. The V717→F mutation is known to alter cleavage specificity of the APP γ-secretase such that the preference for cleavage at the Aβ42 site is increased Thus, Asp2 acts directly or indirectly to facilitate γ-secretase processing of APP at the β42 cleavage site.

Materials

Antibodies 6E10 and 4G8 were purchased from Senetek (St. Louis, MO). Antibody 369 was obtained from the laboratory of Paul Greengard at the Rockefeller University. Antibody C8 was obtained from the laboratory of Dennis Selkoe at the Harvard Medical School and Brigham and Women's Hospital.

APP Constructs used

The APP constructs used for transfection experiments comprised the following

APP	wild-type APP695 (SEQ ID No. 9 and No. 10)
APP-Sw	APP695 containing the Swedish KM→NL mutation (SEQ ID No. 11 and No. 12),
APP-VF	APP695 containing the London V→F mutation (SEQ ID No. 13 and No 14)
APP-KK	APP695 containing a C-terminal KK motif (SEQ ID No. 15 and No. 16),
APP-Sw-KK	APP695-Sw containing a C-terminal KK motif (SEQ ID No. 17 and No. 18),
APP-VF-KK	APP695-VF containing a C-terminal KK motif (SEQ ID No. 19 and No. 20).

These were inserted into the vector pIRES-EGFP (Clontech, Palo Alto CA) between the *Not*1 and *BstX*1 sites using appropriate linker sequences introduced by PCR.

Transfection of antisense oligomers or plasmid DNA constructs in HEK293 cells, HEK125.3 cells and Neuro-2A cells,

Human embryonic kidney HEK293 cells and mouse Neuro-2a cells were transfected with expression constructs using the Lipofectamine Plus reagent from Gibco/BRL. Cells were seeded in 24 well tissue culture plates to a density of 70-80% confluence. Four wells per plate were transfected with 2 µg DNA (3:1, APP:cotransfectant), 8µl Plus reagent, and 4µl Lipofectamine in OptiMEM. OptiMEM was added to a total volume of 1 ml, distributed 200 µl per well and incubated 3 hours. Care was taken to hold constant the ratios of the two plasmids used for cotransfection as well as the total amount of DNA used in the transfection. The transfection media was replaced with DMEM, 10%FBS, NaPyruvate, with antibiotic/antimycotic and the cells were incubated under normal conditions (37°, 5% CO_2) for 48 hours. The conditioned media were removed to polypropylene tubes and stored at –80°C until assayed for the content of Aβ1-40 and Aβ1-42 by EIA as described in the preceding examples. Transfection of antisense oligomers into HEK125.3 cells was as described in Example 7.

Preparation of cell extracts, Western blot protocol

Cells were harvested after being transfected with plasmid DNA for about 60 hours. First, cells were transferred to 15-ml conical tube from the plate and centrifuged at 1,500 rpm for 5 min to remove the medium. The cell pellets were washed with PBS for one time. We then lysed the cells with lysis buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM sodium vanadate and 1% NP-40). The lysed cell mixtures were centrifuged at 5000 rpm and the supernatant was stored at -20°C as the cell extracts. Equal amounts of extracts from HEK125.3 cells transfected with the Asp2 antisense oligomers and controls were precipitated with antibody 369 that recognizes the C-terminus of APP and then CTF99 was detected in the immunoprecipitate with antibody 6E10. The experiment was repeated using C8, a second precipitating antibody that also recognizes the C-terminus of APP. For Western blot of extracts from mouse Neuro-2a cells cotransfected with Hu-Asp2 and APP-KK, APP-Sw-KK, APP-VF-KK or APP-VF, equal amounts of cell extracts were electrophoresed through 4-10% or 10-20% Tricine gradient gels (NOVEX, San Diego, CA). Full length APP and the CTF99 β-secretase product were detected with antibody 6E10.

Results